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Insect appendages and comparative ontogenetics

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Abstract

It is arguable that the evolutionary and ecological success of insects is due in large part to the versatility of their articulated appendages. Recent advances in our understanding of appendage development in *Drosophila melanogaster*, as well as functional and expression studies in other insect species have begun to frame the general themes of appendage development in the insects. Here, we review current studies that provide for a comparison of limb developmental mechanisms acting at five levels: (1) the specification of ventral appendage primordia; (2) specification of the limb axes; (3) regulation and interactions of genes expressed in specific domains of the proximal–distal axis, such as *Distal-less*; (4) the specification of appendage identity; and (5) genetic regulation of appendage allometry.

Keywords: Insect appendages; Appendage primordia; Limb axis specification; *Distal-less*; Appendage patterning; Appendage allometry; Comparative developmental genetics

Introduction

Insects have enjoyed an unparalleled evolutionary success. This has been due, at least in part, to the versatility of their articulated appendages, which allow a wide range of behaviors and novel feeding opportunities. While the basic anatomy of appendages, particularly legs, is well conserved among insect groups, it is also the case that individual taxa have modified their appendages to serve an amazing range of functions. The structure and development of insect appendages have long been an active area of investigation approached from many biological disciplines. In the last decades, genetic and developmental studies in the fruit fly *Drosophila melanogaster* have provided insights into the mechanisms that produce the appendages in this species. This work has been of great value, and has enabled researchers to take a comparative approach to the study of developmental genetics in other insects and arthropods. Admittedly, we are still far from an explanation of biological diversity in which morphology may be unambiguously described by our knowledge of ontogenetic mechanisms. However, by comparing what is known of appendage development in *Drosophila* with that of other species, a more complete understanding of insect appendage development and evolution is slowly emerging.

One of the best-studied developmental regulatory genes has been *Distal-less* (*Dll*), which encodes a helix–loop–helix homeodomain transcription factor and is required for the development of distal limb structures (Cohen et al., 1993; reviewed by Panganiban, 2000). Comparative studies into the molecular mechanisms of limb development were jump-started in the mid-1990s by the remarkable versatility of a broadly cross-reactive antibody produced by Grace Boekhoff-Falk (formerly Grace Panganiban) and her colleagues (Panganiban et al., 1994b) against the *Drosophila* Dll protein. This antibody marks the distal portion of appendages and appendicular derivatives across animal phyla (Panganiban et al., 1997). This surprising result suggested a broad conservation of limb patterning mechanisms. However, not all aspects of limb patterning may be as universal as initially inferred from the widely conserved pattern of *Dll* expression. For example, several recent

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studies (e.g., Angelini and Kaufman, 2005; Jockusch and Ober, 2004; Prpic et al., 2003) have suggested that limb specification mechanisms described for *Drosophila* may not be representative of insects in general.

Despite a wide range of anatomical diversity in the morphology of limbs, all insects must execute the same fundamental processes during appendage development. First, the appendage primordia must be specified. Second, the appendage must be specified as the proper type, or suppressed, according to its position on the body. Third, as a three-dimensional structure, the symmetry of the primordium must be broken into anterior–posterior (AP), dorsal–ventral (DV), and proximal–distal (PD) axes. Fourth, specific regions along the PD axis must then be distinguished in their gene expression to produce anatomically distinct regions. Finally, the size of the appendage relative to the body must be determined. Here, we will review recent advances into the development of the *Drosophila* appendages, as well as relevant data from other species, in order to examine our current understanding of these processes in the development of insect limbs generally.

Insect appendage anatomy

Despite specializations into multiple appendage types, such as antennae, legs, and mouthparts, modern insect appendages are considered to be serially homologous structures that retain anatomical and developmental aspects of their common evolutionary origin (Boxshall, 2004; Snodgrass, 1935). An additional feature of limb evolution in insects is that limbs have been suppressed from the abdomen in a majority of insects.

Arthropods are distinguished from allied phyla, in part, by the synapomorphy of appendage segmentation. The influential entomologist and arthropod physiologist Robert Evans Snodgrass (1935) termed the segments of arthropod limbs "podomeres." The Onychophora are a phylum of lobopodous animals closely allied to the arthropods. However, the limbs of Onychophora have muscle attachments that extend only from the cuticle of the appendage to the body wall, while arthropods also have muscle attachments that interconnect the individual distal podomeres. This allows flexure at the joints of the limb segments and thus a greater range of motion in the appendage.

The most basal segment of the arthropod appendage bears muscle attachments to the body wall, similar to what is seen in the Onychophora. Based on this fact, Snodgrass (1935) considered this proximal "coxopodite" homologous to the entire limb of lobopods, while the distal "telopodite" has been considered an evolutionary innovation of the arthropods, which is subsequently divided into additional distal podomeres. This assignment of homology seems questionable, since Dll protein appears in the distal limbs of both Onychophora as well as arthropods (Panganiban et al., 1997). However, the coxopodite and telopodite do represent distinct regions of gene activity in insects and other arthropods (see below).

Insect dorsal appendages, such as wings, halteres, elytra, and other wing derivatives, have traditionally been considered modifications of the body wall cuticle (Flower, 1964). More recently, however, morphological (Kukalová-Peck and Richardson, 1983) and molecular (Averof and Cohen, 1997) evidence has suggested that they are derived from paraxial outgrowths of the limb coxopodite, such as the endites of primitive insects or the epipods of crustaceans. This issue remains controversial and illustrates a larger evolutionary concern: the degree to which co-option of genetic networks (and molecular markers) has factored into the evolution of novel structures. Here we will not discuss issues related to insect wing evolution and development, but a number of other sources are available for the interested reader (Angelini and Kaufman, in press; Boxshall, 2004; Brodsky, 1994; Jockusch and Ober, 2004). Our remaining discussion will consider the ventral appendages, including the legs, antennae, and mouthparts.

Most insects share the same basic appendage types at different positions along the body (Figs. 1A–F). As noted the ventral appendages of insects primitively consist of a coxopodite and (except for the mandibles) a telopodite of variously modified segmentation. Legs are perhaps the most anatomically conserved of insect appendages. They primitively consist of six podomeres: a proximal coxa, trochanter, femur, tibia, tarsal segment(s), and a distal pretarsus (Fig. 1B). Although in some cases, podomeres may fuse or fail to subdivide, such as in the larval legs of some Holometabola (see below). In the Odonata and some Hymenoptera, the trochanter or proximal femur may also be divided superficially into a "second" trochanter (Daly et al., 1998), but this is likely a secondary modification. The fifth or penultimate podomere is frequently subdivided into as many as five tarsi. Genetic studies in *Drosophila* have suggested that the tarsi and the antennal flagellum are segmented by similar mechanisms (reviewed by Kojima, 2004), supporting their homology. The pretarsi are often modified into hook-like claws and/or pulvilli, which provide traction (Daly et al., 1998).

The insect antenna possesses three true podomeres: the scape, pedicel, and flagellum (Fig. 1A). The flagellum may be secondarily divided into a highly variable number of segments. These flagellar segments lack independent muscle attachments in pterygote insects as well as most basal hexapod lineages (Imms, 1939).

The three pairs of insect gnathal appendages are the mandibles, maxillae, and labial appendages, from anterior to posterior, respectively. The primitive insect mouthpart anatomy is termed "mandibulate" due to the prominence of unjointed chewing mandibles (Fig. 1D). Both systematicists and insect anatomists have used this term: the Mandibulata are a taxon uniting myriapods, crustaceans, and hexapods, in which the mandible is thought to have a common ancestry. Here it is used in the anatomical (rather

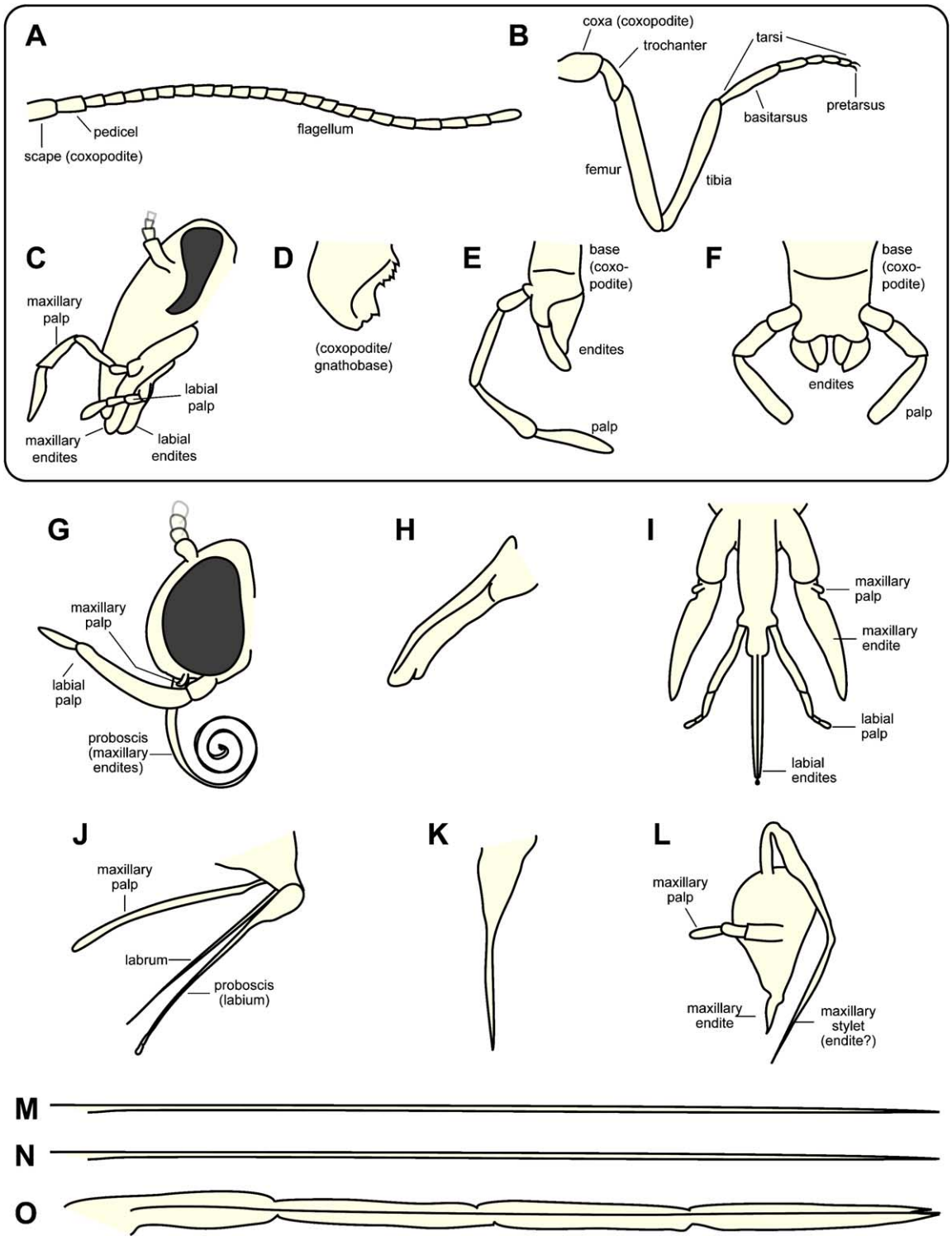


Fig. 1. Anatomy of insect appendages. (A–F) Relatively generalized morphologies of insect appendage types. (A) A filiform antenna, as in Orthoptera. (B) A leg typical of insects, here based on *Drosophila*. (C) The primitive mandibulate insect mouthparts are shown here on the head of a cockroach. (D–F) Individual mandibulate gnathal appendages consist of a pair of (D) chewing mandibles, (E) maxillae, bearing palps and medial endites, and (F) a medially fused labium, also bearing palps and endites. (G–O) However, mouthparts show extensive modification in various insect groups. (G) The head of the lepidopteran *Synanthedon exitiosa* in lateral view. (H) A mandible of the bee *Andrena carlini* (Hymenoptera) modified for shaping wax. (I) Maxillae and labium from a worker bee of *Apis unicolor* (Hymenoptera). (J) Mouthparts of the testes fly *Glossina palpalis* (Diptera, Nematocera). (K) The unpaired mandibular stylet and (L) maxilla of the thrips *Anaphothrips striata* (Thysanoptera). (M) Mandibular and (N) maxillary stylets and (O) labium of the hemipteran *Oncopeltus fasciatus*. (Length has been proportionally reduced here for clarity.) Sketches C, E–J are drawn after Snodgrass (1935); sketches A, D, K–L are drawn after Daly et al. (1998).

than phylogenetic) sense, denoting the primitive insect anatomy of mandibles, maxillae, and labium. Because the mandibles consist of a single podomere, Snodgrass (1928) suggested that the entire mandible was "gnathobasic," with homology to the coxopodite of other appendages and the presumably gnathal surface of the proximal limb segment in trilobites. Studies of *Distal-less* have shown that this gene is normally expressed in the telopodite and no *Dll* expression is found in the mandibles of insects (Panganiban et al., 1994b) or other arthropods (Scholtz et al., 1998), supporting the coxopodite homology of mandibles. Primitively, the maxillae and labial appendages share a similar anatomy, except that the labial appendages are fused midventrally into the labium (Figs. 1E–F). Both appendages consist of large proximal (coxopodite) podomeres bearing two pairs of medial endites. These endites are articulated paraxial outgrowths, which also serve a chewing function in some lineages. The maxillary and labial palps may consist of up to seven segments or podomeres and have been considered homologous to the telopodite of the legs (Snodgrass, 1928). *Dll* is expressed and functionally required in the palps as well as the medial endites (Abzhanov and Kaufman, 2000; Beermann et al., 2001). Mandibulate mouthpart anatomy is found among apterygotes such as the Zygentoma, most hemimetabolous orders, including the Orthoptera, as well as the Holometabola, such as some Coleoptera.

Insect mouthparts have been extensively modified in different lineages as insects have exploited particular food sources (Figs. 1G–O). For example, cyclorrhaphous Diptera, such as *Drosophila*, have drastically reduced mandibular and maxillary appendages, while extensively modifying the labium into a sponging proboscis. Lepidoptera typically feed from the nectaries within flowers, which are reached by a long-coiled "proboscis" derived from the galea, one of the maxillary endites (Fig. 1G). Other lepidopteran gnathal structures are typically reduced, while in some species, such as *Bombyx mori*, adults do not feed and the mouthparts are entirely absent. The maxillary palps of Hymenoptera are drastically reduced, while the glossae and paraglossae, the labial endites, are elongated into what beekeepers have called a "tongue" (Fig. 1I). The mandibles of many higher bees (Hymenoptera) are modified for the task of shaping the wax honeycomb (Fig. 1H), rather than food gathering (Root, 1990). Among the ants (Hymenoptera), the mandibles of many species are modified into weapons for colony defense or hunting prey, or may function as tools to cut and gather leaf matter for raising fungus (Hölldobler and Wilson, 1990). Piercing, sucking mouthparts have evolved independently in several insect lineages (Figs. 1J–O). Nematoceran Diptera, such as mosquitoes and other biting flies pierce the skin of vertebrates with needle-like mouthparts consisting of labrum, hypopharynx, and labium (Fig. 1J). The Siphonaptera (flees and lice) have independently converged on a similar mouthpart anatomy. In thrips (Thysanoptera), the right mandible is suppressed or absent, and the left mandible functions as a piercing stylet (Fig. 1K), as does an endite of the maxillae (Fig. 1L). The mouthparts of Hemiptera are highly derived and modified for suctorial feeding. In this group, the mandibles and maxillae are modified into thin stylets that form channels for fluids (Figs. 1M–N), and the labium is modified into a long jointed appendage, lacking palps (Fig. 1O), which functions to hold and position the stylets. These diverse structures are nevertheless homologous appendages.

Embryonic and larval origins of insect limbs

Ancestrally, the appendages of arthropods arise from embryonic limb buds. This state is retained in ametabolous and hemimetabolous insects, which lack a true metamorphosis. However, in some holometabolous insects, all or some of the adult appendages may be produced from imaginal precursor cells. Our best understanding of this process comes from the cyclorrhaphous Diptera, especially *Drosophila*, where the process is highly advanced and the "imaginal discs" develop as circular epithelial sheets. Imaginal precursors are internalized during larval stages, but remain attached to the ectoderm via a peripodial membrane. These imaginal primordia are set aside during embryogenesis and are subsequently patterned during the larval stages. The discs mature into their adult morphology during the metamorphic pupal stage. During the pupal stage in the Cyclorrhapha, the center of the circular disc telescopes outward to form the distal tip of the appendage while the lateral edges form the proximal portions of the limb. In species where the adult appendages develop from imaginal discs, the corresponding larval appendages may be reduced or represented only by small sensory structures, such as the Keilin's organs of *Drosophila*.

Imaginal disc development is not universal within the Holometabola. As noted, the trend toward imaginal disc development is most advanced in the Cyclorrhapha where all adult appendages are produced from imaginal discs and larvae lack obvious appendages. This has been suggested as an adaptation allowing for rapid development in ephemeral habitats (Svacha, 1992; Truman and Riddiford, 1999). In contrast, only the wings and possibly the genitalia of the Coleoptera, Trichoptera, Neuroptera, and Lepidoptera are derived from imaginal disc-like precursors. In some Hymenoptera, the adult legs and wings also develop from discs. However, whether these cells originate in the embryo, as with *Drosophila* discs, is unknown.

Larvae of such groups may bear well-developed mouthparts, but antennae and legs are typically reduced relative to the adult state. For example, the larval antennae of the red flour beetle *Tribolium castaneum* consist of three segments, while in the adult there are eleven. To a lesser extent, segmentation may also vary between larval and adult stages in hemimetabolous insects: larvae of the milkweed bug

Oncopeltus fasciatus (Hemiptera) have two tarsi, while adult legs have three. Additionally, it has been shown from studies in the silkmoth *B. mori* that the segmentation of larval appendages does not correspond directly to the adult podomeres (Svacha, 1992). In *Drosophila*, the polyploid larval epidermis dies during the pupal stage (Smith and Orr-Weaver, 1991). However, in species such as *Bombyx*, larval structures are not "replaced" by imaginal tissues; rather imaginal histoblasts within the larval appendages maintain developmental continuity to adult appendages (Svacha, 1992).

Establishment of the appendage primordia

The primordia of insect thoracic and gnathal appendages arise from paired populations of cells in the ventrolateral region of the segments in these body regions. At the molecular and genetic levels, *Drosophila* has provided our most complete understanding of the specification of appendage primordia (summarized in Figs. 2A–C). The segment polarity gene *wingless* (*wg*) encodes a secreted Wnt signaling molecule (Nusse and Varmus, 1992), which is required for formation of the embryonic appendage

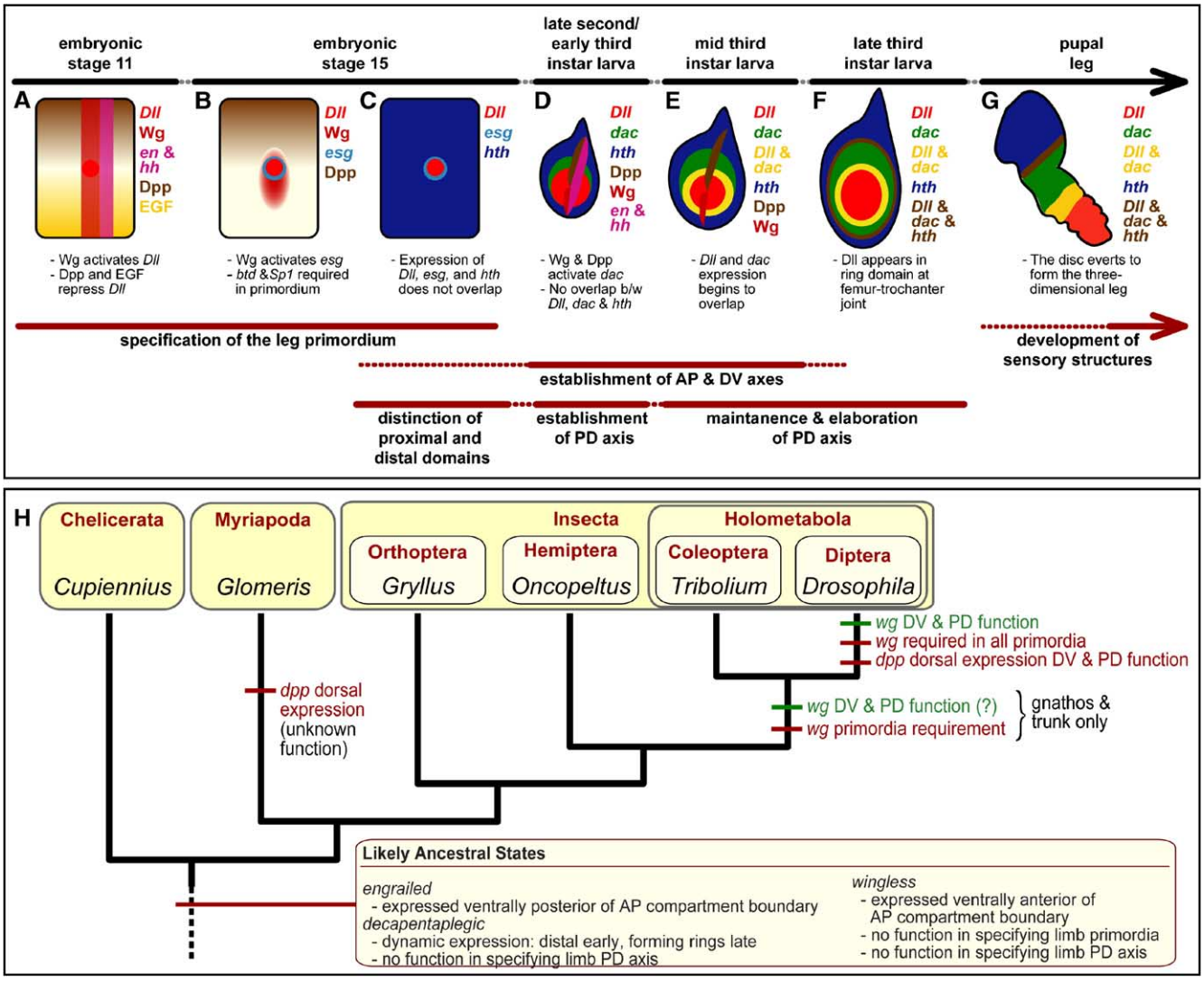


Fig. 2. (A–G) Timeline summarizing important events in the development of the *Drosophila* leg. For clarity, not all genes are shown at any one stage. Those shown in a given diagram are listed to the right of each diagram. (A–C) Early events are depicted in the context of a thoracic body segment, (D–F) while later diagrams focus only on the imaginal discs. (A) At embryonic stage 11, the primordia first become evident, as detected by *Dll* expression. (B–C) By embryonic stage 15, *wg* expression has become localized around the primordium (B), where it is required for proximal identity and expression of *esg* (C). (D) In the late second to early third instar, the leg disc DV and PD axes are established by Wg and Dpp. *dac* expression appears in an intermediate domain, which does not overlap *Dll* or *hth* expression. (E) By the mid third instar, *Dll* and *dac* expression overlaps in the presumptive tibia. (F) Late in the third instar, *Dll* expression appears in a proximal ring overlapping with *hth* and *dac*. (G) During pupal development the disc everts, and *Dll* expression appears in sensory bristles throughout the leg. Panels A–C are based primarily from Kubota et al. (2003). Panels D–F are modified from Abu-Shaar and Mann (1998). (H) A phylogenetic tree of arthropods, showing inferred evolutionary changes in the expression and function of *wg* and *dpp* in limb patterning. It is unclear exactly when in the lineage leading to *Drosophila* that *wg* acquired functions in DV and PD axis specification; therefore, the possible upper and lower limits of this event are indicated on the tree in green.

primordia in *Drosophila* (Simcox et al., 1989). The activity of *wg* is also required for formation of the appendage primordia in *Tribolium*, where *wg* RNAi causes the loss of gnathal and thoracic appendages (Jockusch and Ober, 2004; Ober and Jockusch, submitted). However, the labrum and antennae appear unaffected by depletion of *wg* activity in this species, suggesting that a separate mechanism for the specification of appendage primordia may function in these body segments. In contrast, to the requirement for *wg* in the primordia of these holometabolous insects, all appendage types develop normally in the hemimetabolous insect *Oncopeltus* despite RNA interference of *wg* or its transducer, *pangolin* (Angelini and Kaufman, 2005). Thus, these three species present differing roles for *wg* in the specification of limb primordia, and without additional taxon sampling, it is unclear whether any of these represent the ancestral condition for insect limb development.

In *Drosophila*, Wg protein first appears in a stripe anterior of the parasegment boundary, where it is required to activate expression of *Distal-less* (*Dll*), a gene required for the development of the appendage telopodite (Cohen et al., 1993). In the embryo, *Dll* expression is restricted by the activity of *decapentaplegic* (*dpp*) and epidermal growth factor (EGF) signaling, in the dorsal and ventral ectoderm, respectively (Goto and Hayashi, 1997b).

Dorsal and ventral proximity of Dpp and EGF signaling distinguishes the dorsal (wing or haltere) and ventral (leg) primordia in *Drosophila* (Kubota et al., 2000). In these primordia, expression of the related zinc-finger transcription factors *buttonhead* (*btd*) and *Sp1* is also necessary to allow expression of *Dll* in the leg and sufficient to induce leg identity in the dorsal primordia (Estella et al., 2003).

In the embryonic leg primordium of *Drosophila*, *Dll* expression becomes restricted to cells of the presumptive telopodite, i.e., the femur and more distal podomeres (Gonzalez-Crespo and Morata, 1996). After this point, *Dll* appears necessary and sufficient to identify the distal limb region within the imaginal disc. For example, ectopic expression of *Dll* in proximal cells leads to a nonautonomous duplication of the limb PD axis (Gorfinkiel et al., 1997), while clones lacking *Dll* fail to contribute to the distal region (Campbell and Tomlinson, 1998). The embryonic primordium also requires Wg activity for the specification of proximal leg fates (Kubota et al., 2003). This requirement is separate from the activity of Wg during later larval stages, when it acts to suppress the expression of proximal limb markers and fates (see below).

Suppression of appendage primordia

In insects, the abdominal appendages are suppressed by the Hox genes *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) (reviewed by Hughes and Kaufman, 2002b), but the mechanisms of suppression may differ between species. On the first abdominal (A1) segment, many insects bear gland-like organs called pleuropodia, which are considered to be appendage derivatives. In *Tribolium* (Bennett et al., 1999) and *Oncopeltus* (Angelini et al., in review), *Ubx* is required to modify the development of the A1 appendages to produce pleuropodial structures. When *Ubx* activity is eliminated or reduced, the pleuropodial structures are transformed to legs. These species span the division between holometabolous and hemimetabolous insects (although the hemimetabolous insects are paraphyletic, and *Oncopeltus* represents the sister-group to the Holometabola); therefore, it is likely that *Ubx* has an ancestral role in modifying A1 appendage fate to produce pleuropodia or their derivatives. Modification of the leg developmental program by *Ubx* does not involve the repression of *Dll* in the A1 appendage primordia of *Tribolium* or *Oncopeltus*. Instead, *Ubx* must act downstream of this step to modify appendage identity.

In contrast, *Drosophila* lacks a pleuropodial structure, and in this species *Ubx* acts directly to repress *Dll* in the first abdominal segment (Castelli-Gair and Akam, 1995; Vachon et al., 1992), a function shared with *abdominal-A*, which represses *Dll* expression and limb development on more posterior abdominal segments of all three species (Angelini et al., in review; Carroll et al., 1995; Stuart et al., 1993). The *Dll*-repressing activity of *Ubx* in *Drosophila* is likely derived, but it is unclear at what point in the fly's lineage this activity appeared.

Among some groups, such as the basal hexapod Collembola and larval Lepidoptera, functional appendages are present on the abdomen. In these species, embryonic limb primordia in the abdomen are consistently marked by *Dll* expression (Palopoli and Patel, 1998; Panganiban et al., 1994a; Warren et al., 1994). However, the underlying molecular networks in each of these lineages seem to act differently to permit abdominal *Dll* expression and the development of appendage primordia. In the collembolans *Folsomia candida* and *Xenylla grisea*, Dll and Ubx/Abd-A proteins co-occur in the same cells, suggesting that unlike the insects studied, neither of these Hox genes represses *Dll* in the Collembola.

Interestingly, while the Collembola are most often noted for the presence of abdominal appendages, not all abdominal segments bear limbs. In *Folsomia* and *Xenylla*, appendages are absent from A2, A5, and more posterior segments (Palopoli and Patel, 1998). It is entirely unknown what genes act to repress appendages on these segments or to distinguish between the distinct identities of the A1, A3, and A4 abdominal appendages.

In larvae of the moth *Manduca sexta*, Ubx and Abd-A proteins are absent from areas of Dll accumulation in abdominal segments A3–A6, where larval prolegs form. The otherwise uniform domain of Hox gene expression in this species shows "holes" where Dll protein accumulates (Warren et al., 1994). Interestingly, in *B. mori*, another lepidopteran with similar larval prolegs on A3–A6, deletions of *Ubx* and *abd-A* result in ectopic prolegs on abdominal segments A1–A8 (Ueno et al., 1992). Thus,

there is an apparent requirement for *Ubx* and *abd-A* to suppress appendage development on segments A1–A2 and A7–A8 in *Bombyx*, where "holes" do not normally form in the Hox gene expression domain. Therefore, Ubx and abd-A are evidently capable of *Dll* repression. Evidently, they do not do so in A3–A6 because they are actively repressed in the "holes" of those segments, allowing the appendage primordia pathway to be activated. Put differently, it appears that the regulatory interactions between *Dll* and these Hox genes in the Lepidoptera have not been altered relative to other insects; rather, other factors regulating Hox expression have partitioned cells expressing *Ubx* or *abd-A* from those expressing *Dll* in the abdomen.

What then can account for the observed difference between the Hox–Dll relationship in the collembolan and the moths? As discussed above, it seems to be an ancestral function for *Abd-A* in insects to repress *Dll* expression and appendage development. In contrast, Abd-A expression often overlaps with appendage development in non-insect arthropods, including the basal hexapod collembolans. It is likely that, after the divergence of the Collembola and Insecta, the insect Abd-A protein may have acquired repressive functions, or specifically repressive binding sites for Abd-A may have appeared in the regulatory regions of the insect *Dll* locus, in conjunction with the evolution of a limb-less abdomen in this lineage. Because abdominal prolegs are a secondary characteristic of *Manduca* and other Lepidoptera, the holes in *Abd-A* expression appear to be an adaptation allowing the expression of *Dll* and the appearance of prolegs.

The results of Ronshaugen et al. (2002) lend credence to a portion of this evolutionary scenario. These investigators noted that, in many crustaceans, limbs are formed on all of the trunk segments, and as in the Collembola *Ubx* and *Dll* are coexpressed. They cloned the *Ubx* ortholog from the crustacean *Artemia fransicana* and expressed it in *Drosophila* where the *Artemia* protein was unable to repress *Dll* expression or prevent the initiation of appendage development. They were further able to show that this failure to repress *Dll* was associated with a specific difference in the amino acid sequence of the *Artemia* protein relative to the *Drosophila* ortholog. The conclusions of this study should be treated cautiously due to the large evolutionary distance between *Artemia* and *Drosophila* (at least 409 million years). However, it is possible that a similar difference may exist in the Abd-A proteins of collembolans and insects.

Specification of the limb axes

One of the most general principles of developmental biology is that the tissues of multicellular organisms must establish axial polarity in order to perform their organismal function. Animal appendages have been a classical system in which to study axis specification, and much is known of this process in *Drosophila*. However, comparative studies demonstrate that this mechanism is not universal (see Fig. 2H).

As noted, the *Drosophila* appendage primordia are derived from cells at the parasegmental boundary, and they appear to inherit their anterior–posterior (AP) polarity from the segmentation genes involved in germband segmentation. In the imaginal leg disc of *Drosophila*, as in the germband, the homeodomain transcription factor *engrailed* (*en*) and the secreted signal encoded by *hedgehog* (*hh*) are expressed posterior of the AP compartment boundary. The activity of these genes is thought to maintain AP polarity in the imaginal discs, since null mutant clones in the imaginal wing disc can cause the transformation of cells to the identity of the opposite compartment as well as mirror-image duplication of the appendage (e.g., Tabata et al., 1995). Hedgehog signaling activates the expression of *wg* ventrally, on the anterior side of the AP boundary and *dpp* dorsally on the anterior side (Diaz-Benjumea et al., 1994). Dpp and Wg then act in a mutually repressive manner to define the dorsal and ventral territories of the disc, respectively (Theisen et al., 1996).

By the third instar, *Drosophila* imaginal discs become more elaborately patterned in the proximal–distal (PD) axis (Figs. 2D–F). This positional information is imparted by Wg and Dpp (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). *wg* and *dpp* are expressed in radial stripes, from the disc center to its periphery, anterior of the AP compartment boundary. The activity of both signals is required to activate genes at distal positions of the PD axis, such as *Dll*, while they inhibit expression of proximal markers, such as *homothorax* (*hth*). In clones lacking the activity of *wg* or *dpp*, *Dll* is down-regulated nonautonomously and in a time-dependent manner (Lecuit and Cohen, 1997). Therefore, Wg and Dpp signals appear to diffuse from ventral and dorsal regions. They overlap at higher concentrations in the center of the disc, the presumptive distal tip, where they cooperatively activate distal targets such as *Dll*.

In all arthropod groups examined, *engrailed* is expressed in a conserved pattern, on the posterior side of the parasegmental boundary (e.g., Damen, 2002; Hughes and Kaufman, 2002a; Ingham and Martinez-Arías, 1992; Patel et al., 1989). In species where the appendages develop from limb buds, this expression continues across the germband into the limb buds, and *wg* expression is conserved in segmental stripes abutting *en* expression on the anterior of the parasegment boundary (Angelini and Kaufman, 2005; Hughes and Kaufman, 2002a; Miyawaki et al., 2004). However, the expression of *dpp* orthologs in the limbs of non-model species does not resemble that of the *Drosophila* imaginal leg disc. In the Hymenopteran *Athalia rosae* (Yamamoto et al., 2004), *Tribolium* (Sanchez-Salazar et al., 1996), *Oncopeltus* (Angelini and Kaufman, 2005), and the grasshopper *Schistocerca americana* (Jockusch et al., 2000), early expression of *dpp* appears throughout the limb buds. As the limb buds elongate, rings of expression are

formed at or just proximal of the distal tip. In late stages, additional weaker rings of *dpp* expression appear more proximally in some species. These data have suggested that while AP axis specification may be conserved, Dpp signaling was unlikely to act in establishment of the DV or PD limb axes ancestrally (Fig. 2H).

Recent functional evidence also suggests that Wg and Dpp signaling in other insects may not specify PD domain genes as in *Drosophila*. Jockusch and Ober (2004; submitted for publication) have used RNAi to test the function of *wg* and *dpp* during the embryonic development of *Tribolium*. Suppression of *wg* eliminates the gnathal and thoracic appendages, although it was not determined whether this is due to a requirement for *wg* activity in the specification of the appendage primordia or in the maintenance of the AP compartments or limb axes. Interestingly, no effect was observed in the anterior-most appendages (the labrum and antennae). These results demonstrate that a requirement for *wg* in the development of most appendages is conserved in *Tribolium* and *Drosophila*. In contrast, RNA interference of *dpp* did not cause defects in appendages, although it did prevent the development of dorsal embryonic tissues. This evidence supports the predictions of earlier expression studies that *dpp* may not function in DV or PD axis specification except in *Drosophila*.

A recent study from the cricket *Gryllus bimaculatus* has shown that RNA interference of *armadillo*, the transducer of canonical Wnt signaling, produces embryonic defects in germband elongation and segmentation, but embryos still bear appendages (Miyawaki et al., 2004). We have recently reported a similar analysis in *Oncopeltus* (Angelini and Kaufman, 2005), where RNA interference of *wg* and another Wnt signaling component, *pangolin*, produces defects in the eye and in germband elongation and segmentation, but no appendage defects were found.

Gryllus represents a fairly basal insect group, while *Oncopeltus* represents the sister-clade of the Holometabola (Wheeler et al., 2001). Therefore, it is possible that the genetic network described for the specification of limb axes in *Drosophila* has been extensively modified from the ancestral state, sometime after the divergence of Diptera from Coleoptera. However, because appendage development appears to have differing requirements for *wg* in *Drosophila*, *Tribolium*, *Oncopeltus*, and *Gryllus*, limb axis specification also may be highly variable among insects. Fig. 2H presents the evolutionary scenario as described by the available data, but it is obvious that much greater sampling of taxonomic groups will be necessary for a complete understanding of the evolutionary history of limb axis specification.

Genes specifying proximal–distal limb domains

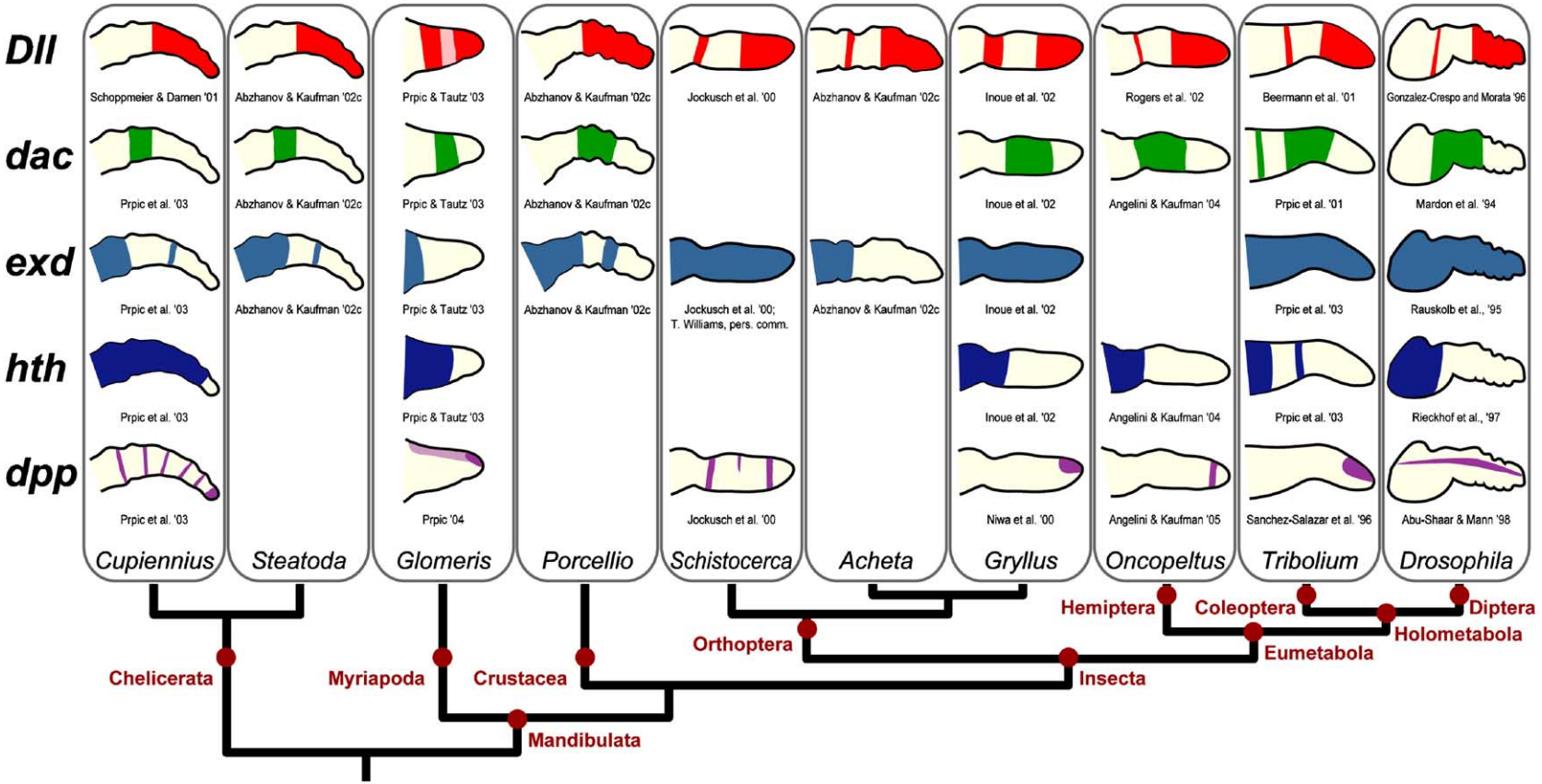
Genes controlling the identity of specific PD regions of the limb act in an analogous manner to the gap genes of the embryonic germband in that loss of their activity results in the loss of structures, rather than their transformation. This is reflected by the fact that genes such as *Dll* and *dac* are positively required for the growth and development of the regions in which they are normally expressed. While this may be due in part to the activation of cell proliferation, it has been shown that in *Drosophila* the PD domain genes act principally to segregate cells along the PD axis (Wu and Cohen, 1999). Cells of specific domains are not identified by their lineage, but rather as a result of their surroundings (European vs. American plan *sensu* P. Lawrence). As described above, the deployment of the genes that regionally specify the limb PD domains results from their differential regulation by Wg and Dpp signals in *Drosophila*. These ligands cooperatively activate distal domain genes, while repressing proximal domain genes.

Much of the work on appendage development in *Drosophila* has focused on the leg discs. Therefore, we will initially focus on data from the legs of *Drosophila* and other insects and subsequently discuss what is known about other appendage types.

Distal-less and *dachshund*

Genetic screens carried out in *Drosophila* have identified mutations that eliminate specific podomeres along the PD axis of the leg (e.g., Ashburner et al., 1990; Sato, 1984). Strong *Distal-less* (*Dll*) alleles are embryonic lethal, but hypomorphic mutations allow the recovery of adult flies with truncated appendages. In the legs of such adults, podomeres distal of the femur are eliminated (Cohen and Jürgens, 1989b). Mutations in *dachshund* (*dac*) result in stubby legs missing the tibia, but not the distal most structures. Hence, the gene is named for a stocky—but certainly not legless—breed of dog (Mardon et al., 1994).

Among insects, *Distal-less* expression patterns have proven to be very consistent in the species examined by antibody staining or in situ hybridization (see Fig. 3 for a summary of expression patterns and phylogenetic relationships of the most extensively studied species). These species include *T. castaneum* (Beermann et al., 2001), *O. fasciatus* (Rogers et al., 2002), *S. americana* (Jockusch et al., 2000), and the crickets *Acheta domesticus* (Abzhanov and Kaufman, 2000) and *G. bimaculatus* (Inoue et al., 2002). As described above, *Distal-less* expression is one of the earliest markers of the imaginal leg disc primordia in *Drosophila* (Cohen et al., 1993). Early expression is typically broad, but always includes the distal tip. As the limb grows, *Dll* expression becomes progressively restricted to more distal cells (Figs. 2D–G show the *Drosophila* pattern). *Dll* expression stabilizes in the cells of the distal tibia, tarsi, and pretarsus, while a separate domain of *Dll* expression appears in the leg at the trochanter–femur joint (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). These two domains of insect *Dll* expression have been described as a proximal



Additional Expression Data

- *Dll* in other insects
  - *Manduca* (Lepidoptera; early) - Zheng et al. '99
  - *Precis* (Lepidoptera; early) - Panganiban et al. '94
  - *Thermobia* (Zygentoma) - Rogers et al. '02
  - *Lepisma* (Zygentoma) - Mittmann & Scholtz '01
  - *Folsomia, Xenylla* (Collembola) - Palopoli & Patel '98
- *Dll* in phyllopodous crustaceans
  - *Artemia, Mysidopsis* - Panganiban et al. '95
  - *Daphnia* - Shiga et al. '02
  - *Nebalia, Triops* - Williams '98
  - *Sacculina* - Mouchel-Vielh et al. '02
  - *Thamnocephalus, Triops* - Williams et al. '02
- *Dll* in other arthropods and allies
  - *Argiope* (Arachae) - Panganiban et al. '95
  - *Limulus* (Xiphosura) - Mittmann & Scholtz '01
  - *Peripatopsis* (Onychophora) - Panganiban et al. '97
- *dpp* in other insects
  - *Athalia* (Hymenoptera) - Yamamoto et al. '04

Fig. 3. Diagrammatic representations of gene expression data for *Distal-less* (*Dll*, red), *dachshund* (*dac*, green), *extradenticle* (*exd*, cyan), *homothorax* (*hth*, dark blue), and *decapentaplegic* (*dpp*, violet) from the developing legs of various arthropods. Dynamics of gene expression have been necessarily ignored in this figure, and diagrams are meant to depict final patterns of expression. However, in instances where earlier stages may be informative to the consideration of homologous developmental roles, early expression is indicated by areas of semi-transparent color. An effort has been made to be inclusive of all relevant studies published to date. The phylogenetic relationships between species are based on the conclusions of Giribet et al. (2001; class-level), Wheeler et al. (2001; ordinal-level), and Flook et al. (1999; within Orthoptera) (Mouchel-Vielh et al., 2002; Niwa et al., 2000; Panganiban et al., 1995; Prpic, 2004; Shiga et al., 2002; Williams, 1998; Zheng et al., 1999).

"ring" and distal "sock" (Panganiban et al., 1994b). In *Drosophila*, the ring appears independently (Panganiban, 2000), but in *Gryllus* (Inoue et al., 2002), *Schistocerca*, and *Tribolium* (Jockusch et al., 2004), the separate domains are produced when *Dll* expression is lost from intermediate cells.

Analysis of *Dll* mutations in *Tribolium* (Beermann et al., 2001) and *Dll* RNAi in *Oncopeltus* (Angelini and Kaufman, 2004) has revealed similar loss-of-function phenotypes in which the legs are truncated distal of the femur. While *Dll* function has been poorly sampled among insects, data from other arthropods suggest a phylogenetically broad functional conservation of *Dll*. RNA interference of *Dll* in embryos of the spider *Cupiennius salei* has truncated most of the telopodite from limb buds (Schoppmeier and Damen, 2001). In the isopod crustacean *Porcellio scaber*, Dll accumulation diminishes in the first thoracic appendages as they undergo a normal developmental transformation into reduced maxillipeds, which is apparently accomplished by the deletion of distal elements of the transforming appendage (Abzhanov and Kaufman, 1999, 2000).

The intermediate domain of the leg is marked by *dachshund* in *Drosophila* (Mardon et al., 1994), and well-conserved expression patterns have been reported for *dac* orthologs in *Tribolium* (Prpic et al., 2001), *Oncopeltus* (Angelini and Kaufman, 2004), *Gryllus* (Inoue et al., 2002), and *Acheta* (Abzhanov and Kaufman, 2000). Expression of *dac* appears later than *Dll* in a ring proximal to the *Dll* domain with little overlap (Fig. 2D). Later *dac* expression encompasses intermediate regions of the leg, from the femur through the basitarsus (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997). At this stage, *Dll* and *dac* expression partially overlap across their domains, and both gene products occur in cells of the distal tibia and basitarsus (Fig. 2E). Comparative functional data are more limited for *dac*. However, it has been shown that *dac* RNAi eliminates the tibia in *Oncopeltus* (Angelini and Kaufman, 2004), suggesting conservation of the *dac* gap gene function in eumetabolic insect limbs.

Distal-less and *dachshund* in sensory organ development

Dll and *dac* are also expressed in separate and complex subsets of neurons in the developing nervous system (Kaphingst and Kunes, 1994; Mardon et al., 1994), which is also seen for other homeodomain transcription factors (e.g., Heuer and Kaufman, 1992). Thus, it is not entirely surprising to find that *Dll* and *dac* are also expressed in many sensory structures of the appendages. For example, the proximal ring of *Dll* expression in *Drosophila* correlates with the location of a group of campaniform sensillae in adults, and *Dll* is expressed in and required for the development of bristles throughout the legs, antennae, and wings (Gorfinkiel et al., 1997). Moreover, it has been shown that *Dll* expression along the wing margin is autonomously required for activation of the proneural gene *acheate* in ventral sensory bristles (Campbell and Tomlinson, 1998). Similarly, loss of *Dll* or *dac* activity in the genital disc of *Drosophila* eliminates sensory bristles from the analia and genitalia (Gorfinkiel et al., 1999). Thus, it appears that *Dll* may be required generally for the specification and/or development of appendage-associated sensory structures in *Drosophila*.

This phenomenon is not restricted to flies, as it has also been observed in other arthropods. For example, Dll protein accumulation correlates with sensory organs on the mouthparts and terminalia of the apterygote insect *Lepisma saccharina* (Mittmann and Scholtz, 2001). In the mandibles of this species, specific sensory cells are the only nuclei that stain for Dll. Similarly, in the millipede *Glomeris marginata*, embryonic expression of *Dll* appears in presumptive sensory organs of the maxilla and mandible (Prpic and Tautz, 2003). Expression of *dac* in this species also correlates with these sensory organs, while it is not expressed in an intermediate domain in these mouthparts. The dual role of *dac* in sense organs and intermediate limb elements is not unique to the legs. It is also expressed in the antennae of *Glomeris*, in an intermediate ring and in four distal domains correlating with organs called sensory cones (Prpic and Tautz, 2003). This apparent duality is not restricted to the insects and myriapods. In the branchiopod crustaceans *Thamnocephalus platyurus* and *Triops longicaudatus*, Dll protein appears in the distal regions of all limb branches at early stages. However, in juveniles, Dll expression also appears in cells throughout the limbs at the base of bristle-like setae, which likely have a sensory function (Williams et al., 2002). The limbs of the primitive aquatic chelicerate *Limulus polyphemus* also accumulate Dll protein in distal regions early. However, at later stages, Dll protein correlates with developing mechanoreceptors and sensory neurons in the proximal legs, book gill opercula, and dorsal body surface (Mittmann and Scholtz, 2001). Thus, the limb podomeric function and sensory element roles of *Dll* and *dac* are likely conserved across the Arthropoda and can be considered ancestral functions in the group.

The dual roles of *Dll* and *dac* in sensory structures and limb development give rise to an important evolutionary question: Which function is ancestral? From data on Dll patterns in crustaceans and insects, Williams et al. (2002) have proposed that the function of *Dll* in sensory structures predisposed (i.e., exapted) it to the evolution of its role as a PD domain gene. By promoting growth at the base of mechanosensors, alone or in clusters, *Dll* would be capable of producing an outgrowth. Later in evolutionary history, as animals evolved what we recognize as appendages, the specification of this outgrowth necessarily became heterochronically earlier than the specification of sensory organs.

Interestingly, it appears that *Dll* may also be expressed in outgrowths that are neither appendicular (i.e., not homologous to the ventral or dorsal appendages) nor obviously

sensory: Moczek and Nagy (2005) have recently shown that in sexually dimorphic horned beetles of the genus *Onthophagus*, Dll accumulates in "distal" regions of the male pupal horn, although not in corresponding regions of the female. Thus, it appears that *Dll* and perhaps other appendage patterning components have been co-opted in this genus to promote outgrowth in an axial manner. Furthermore, because dimorphism and horn morphology vary widely among *Onthophagus* species, Moczek and Nagy suggest that *Dll* and other appendage-patterning genes may be highly flexible in horn development and evolution. This flexibility, demonstrated by this example of co-option, implies that the functions of *Dll* in sensory development and growth may be separable at least in some evolutionary contexts.

Genes of the proximal limb domain

Several genes are expressed in the proximal domain, or coxopodite, of insect appendages. The proximal domain of the *Drosophila* leg primordia is evident in the embryo as early as stage 13, where the gene product of *escargot* (*esg*) appears in a domain surrounding Dll protein accumulation (Goto and Hayashi, 1997b; Fig. 2C). Initially (before stage 14), *wg* activity is required for the proximal expression of *esg* (Kubota et al., 2003) and therefore presumably for specification of the proximal domain. Esg overlaps with Hth in the proximal leg primordia, but does not appear in the body wall (Kubota et al., 2003). As the leg disc develops, the proximal region maintains expression of *hth* and *esg*, while expression of *Dll* and *dac* is excluded (Abu-Shaar and Mann, 1998; Kubota et al., 2003). This region of proximal gene activity corresponds to the coxa and trochanter of the adult leg.

The most extensively studied proximal genes are the homeodomain transcription factors *homothorax* (*hth*) and *extradenticle* (*exd*). Expression of *exd* and *hth* is also found in the thoracic body wall (Rauskolb et al., 1995; Rieckhof et al., 1997). Exd protein appears ubiquitously in the leg discs of *Drosophila* but requires the presence of Hth for activity. Only Exd contains a nuclear localization sequence, and Exd and Hth must bind one another in order for Hth to be imported into nuclei where they act as heterodimeric transcriptional regulators (Abu-Shaar and Mann, 1998; Rieckhof et al., 1997). In *Gryllus*, it has also been shown that these proteins become nuclear only where they co-occur in cells (Inoue et al., 2002), implying a functional conservation of these protein interactions.

In the embryonic leg buds of the beetle *Tribolium* (Prpic et al., 2003), the milkweed bug *Oncopeltus* (Angelini and Kaufman, 2004), and the orthopteran *Gryllus* (Inoue et al., 2002), *hth* appears in patterns similar to *Drosophila* in proximal podomeres (Fig. 3). If we consider that a similar proximal expression pattern has been reported for *hth* in the myriapod *Glomeris* (Prpic and Tautz, 2003), then this pattern is likely to be the ancestral state of *hth* expression in insects. However, in the spider *C. salei*, a pair of *hth* paralogues is both expressed broadly along the PD axis of the prosomal walking legs. Expression of *hth* has not been examined in Crustacea, and it remains unclear what the ancestral state of expression may be for all arthropods.

The evolutionary history of *exd* appears to be more complex. In *Tribolium* (Prpic et al., 2003), *Gryllus* (Inoue et al., 2002), and *Schistocerca* (Jockusch et al., 2000), *exd* is expressed throughout the developing legs, but Exd protein is only localized to nuclei proximally, where it co-occurs with Hth (Fig. 3). Interestingly, in the non-insect arthropod species for which Exd accumulation has been examined, the spiders *C. salei* (Prpic et al., 2003) and *Steatoda triangulosa*, and the isopod crustacean *P. scaber* (Abzhanov and Kaufman, 2000), *exd* orthologues are expressed proximally, rather than throughout the limbs. Prpic et al. (2003) have suggested that the reciprocal patterns of *exd* and *hth* expression seen in spiders and insects such as *Drosophila* may represent two alternate evolutionary modifications from an ancestral state in which both genes were expressed proximally, such as exists in the myriapod *Glomeris*. While plausible, this conclusion must be regarded as tentative before more species are sampled, particularly among more diverse myriapods and crustaceans.

Interestingly, this alternative arrangement is also found within the insects. In *A. domesticus* (Abzhanov and Kaufman, 2000), Exd protein is only detected in proximal podomeres of the leg buds. In two other orthopterans, *Schistocerca* and *Gryllus*, Exd appears throughout the limbs, and since *Schistocerca* occupies a more basal position within the Orthoptera (Flook et al., 1999), ubiquitous expression seems ancestral for this order. This implies that expression of *exd* expanded throughout the limbs within the lineage leading to the insects. However, the apparently secondary proximal restriction in *Acheta* is a powerful argument for more extensive taxonomic sampling.

The specification of cell fates in the proximal domain appears to be more complex than in more distal regions. In *Drosophila*, cell clones ectopically expressing *hth* in distal regions of the leg disc are capable of repressing *Dll* and *dac*, and these clones do not mix with distal cells. Instead, they migrate into the proximal region or delaminate into the mesoderm of adult legs (Wu and Cohen, 1999). Clones lacking *hth* migrate out of the leg proper into regions of the disc that will give rise to the body wall. Therefore, *hth* is necessary and sufficient for the segregation of proximal leg cells within the disc. However, when leg discs are produced lacking *hth* activity throughout, proximal podomeres are not deleted. Instead, the coxa, trochanter, femur, and tibia fuse, and genes controlling the segmentation of these podomeres are misexpressed (Casares and Mann, 2001). Importantly, bristle patterns indicative of tibia and femur can be seen in the *hth*-null legs, demonstrating that cells retaining the identity of these podomeres are present at roughly the proper PD locations. Therefore, while *hth* activates leg

segmentation and distinguishes proximal leg cells from cells of the body wall and distal leg, it is not required to specify proximal identity per se in those cells. Through *hth* RNAi, a similar leg phenotype has been produced in *Oncopeltus*, a species in which appendages develop from limb buds rather than imaginal discs, indicating that this function of *hth* is apparently conserved between these developmental modes (Angelini and Kaufman, 2004).

The contrasting roles and loss-of-function effects of the proximally and distally acting genes show that, while identity and cell segregation in the distal and intermediate domains of the leg are provided by a single gene, *Dll* or *dac*, respectively, these functions are separated in the proximal domain. Whereas *hth* provides for cell segregation, the specification of proximal cell identity may be accomplished by *teashirt* (*tsh*) (Wu and Cohen, 2000). This zinc-finger transcription factor is expressed in a pattern overlapping with *hth* in the proximal leg domain. Clones lacking *tsh* activity show up-regulation of *Dll*, and *tsh* mutant adults eclose with a reduced coxa and trochanter. These podomeres also lack sensory organs indicative of these two podomeres, suggesting they lack proximal identity. However, it has not been demonstrated through ectopic expression whether *tsh* expression is sufficient to impart proximal fate in distal domains.

Interactions between the PD domain genes

The distal, intermediate, and proximal domains of the leg are maintained through the interactions of genes such as *Dll*, *dac*, and *hth*. Early in the development of the *Drosophila* leg disc, before expression of *dac* appears, *Dll* represses proximal identity, as marked by *esg* (Kubota et al., 2003). Discs at this stage transplanted into third instar larvae produce only body wall and tarsi (Schubiger, 1974), suggesting that intermediate structures are not yet specified. Lineage tracing has shown that cells of the intermediate podomeres are typically born in the proximal domain. They sort into intermediate regions, lose expression of the proximal marker *tsh*, and activate expression of *dac* and/or *Dll* in response to the higher levels of Wg and Dpp at more distal positions (Weigmann and Cohen, 1999).

Similar interactions define distal, intermediate, and proximal domains later in the *Drosophila* leg disc. As described above, distal clones ectopically expressing *hth* are sufficient to autonomously inhibit the expression of *dac* and *Dll* (Wu and Cohen, 1999), although it has been shown that this repression is mediated by *tsh* (Dong et al., 2001). Similarly, proximal clones in the leg which ectopically express *Dll* do not express *hth* and sort to more distal positions or out of the disc (Wu and Cohen, 1999), while, conversely, Hth appears in distal clones lacking *Dll* or *dac* (Dong et al., 2001). Furthermore, clonal analysis has revealed a similar mutual antagonism between *Dll* and *dac* in the leg disc (Dong et al., 2001).

Comparative data on the interactions of PD domain genes are very limited. In the milkweed bug *Oncopeltus*, epistatic interactions between *Dll*, *dac*, and *hth* have been tested in the legs through the examination of gene expression in RNAi backgrounds (Angelini and Kaufman, 2004). Such experiments lack the sensitivity of clonal analysis, but identified most of the repressive interactions known for these genes in *Drosophila*. This suggests, at least, that the interactions known from *Drosophila* are not unique to the derived context of imaginal discs. As discussed in the preceding section, the expression patterns of *Dll*, *dac*, and *hth* are conserved in the legs of all sampled insects. While coexpression has been tested rigorously by confocal microscope in only a few species (*Gryllus*, Inoue et al., 2002; *Schistocerca*, Jockusch et al., 2000), it is plausible that distinct boundaries are maintained by conserved repressive interactions.

Late in development, mutually repressive interactions may be relaxed in some areas. For example, Dll, Dac, and Hth overlap in a narrow ring of cells in the distal trochanter of the late third instar leg disc in *Drosophila* (Abu-Shaar and Mann, 1998). Distal rings of *hth* or *exd* expression have been reported at late stages in *Tribolium* (Prpic et al., 2003) and *Acheta* (Abzhanov and Kaufman, 2000), while *dac* gains a late proximal domain of expression in the legs of *Tribolium* (Prpic et al., 2001). Furthermore, as we will discuss in the next section, interactions between these genes are specific to individual appendage types. Therefore, the interactions of PD domain genes vary depending on temporal and tissue contexts. As we will also discuss below, it appears that these interactions may also be evolutionarily labile.

Specification of limb identity

Across the insects, PD domain genes, such as *Dll*, *dac*, and *hth*, are expressed in most appendage types. These genes typically vary in the their domains of overlap and interactions in different appendage types, but as we have already discussed, they are consistently required for the development of structures at particular PD levels. How then are specific appendage types distinguished?

The specification of appendage identity in the antennae, proboscis, and legs of *Drosophila* primarily involves the action of the Hox genes but also in part relies on the modulation of the PD domain genes. How the evolution of novel appendage anatomy has correlated with alterations in the patterns and interactions of these genes is less well understood. The limited amount of comparative data currently available makes it difficult to generalize. Nevertheless, based on the apparent conservation of the PD domain genes, it remains a possibility that alterations in their domains of expression and interactions may play a role in the evolution of some morphology. We will review what is known about the most illustrative examples: the specification of antennae and mouthparts in various species.

Antennae

As noted, the primary identifiers of appendage type in the insects are the Hox genes. However, the intermediate regulatory events that impart identity and modify expression and interactions of the PD domain genes are less well understood. The most extensively studied instance of appendage fate specification is that of antennae and legs in *Drosophila*.

In the *Drosophila* thorax, the Hox gene *Antennapedia* (*Antp*) is required for leg identity (Struhl, 1982). Antp is expressed throughout the early leg discs, where it prevents the coexpression of *Dll* and *hth* (Emerald and Cohen, 2004) through an unknown mechanism. Later, *Dll* acts to suppress *Antp* in distal regions via *spineless* (*ss*) (Emerald and Cohen, 2004). Therefore, *Antp* does not act directly to specify leg identity, but does so through modification of the PD domain genes *Dll* and *hth*. Notably, the Hox genes are not expressed in the antennal disc and in this context, *Dll* and *hth* are expressed over a broad overlapping region of the PD axis and do not repress one another as they do in the leg. It is this overlap of *Dll* and *Hth* expression that is required for antennal identity (Dong et al., 2000). Early reports identified alleles of *Dll* by the name *Bristle-on-arista* due to the fact that hypomorphic alleles are associated with the presence of ectopic leg-like bristles on the antennae (Sato, 1984; Sunkel and Whittle, 1987). Subsequently, it has been shown that Dll and Hth proteins cooperate to activate several antennal target genes, including *spalt*, which is required for development of Johnston's organ (Dong et al., 2003) in the pedicel (a2 in *Drosophila* nomenclature), and *ss*, which is necessary for antennal development (Dong et al., 2002; Duncan et al., 1998). The paralogous genes *distal antenna* (*dan*) and *distal antenna-related* (*danr*) are activated by *ss* and appear to be responsible for antennal identity in the disc (Emerald et al., 2003). From this example, one can clearly see the interweaving of the Hox and PD pathways in the specification of appendage identity.

In other insects, several aspects of antenna specification appear to differ. The most immediate gene in the *Drosophila* antennal specification hierarchy, *dan/danr*, appears to lack orthologs in the genomes of non-dipteran species, at least as identifiable by NCBI BLAST. While a possible *dan* ortholog is present in the mosquito *Anopheles gambiae*, this gene has not been studied. *Dll* hypomorphic alleles in *Tribolium* cause truncation of the antennae, but no transformation to leg (Beermann et al., 2001). In *Oncopeltus*, *Dll* RNAi produces a similar antennal truncation phenotype without transformation (Angelini and Kaufman, 2004). This suggests that, in *Oncopeltus* and *Tribolium*, *Dll* and *hth* do not cooperate to specify antennal identity, and thus unique aspects of antennal development in *Drosophila* appear to be derived and may be related to the derived aristate morphology of the brachyceran antenna.

Interestingly, in the embryonic antennae of Orthoptera, domains of Dll and nuclear Exd (n-Exd) accumulation do not resemble those of eumetabolic insects. In *Schistocerca*, only a narrow intermediate region of overlap exists for Dll and n-Exd (Jockusch et al., 2004), while domains appear non-overlapping in the *Acheta* antennae (Abzhanov and Kaufman, 2000). Based on comparisons to serially homologous appendages, this state has been suggested as ancestral (Jockusch et al., 2004). Unfortunately, *hth* expression has not been reported from the antennae of Orthoptera. However, it seems that overlap in *Dll* and n-Exd appeared in the lineage leading to the Eumetabola, but acquire a cooperative role in identifying antennae in the lineage leading to *Drosophila* sometime after its divergence from the lineage of *Tribolium*.

Mouthparts

The mouthparts of insects are ventral appendages sharing serial homology to the antennae and legs. Specific Hox genes are known to specify mouthpart identity in *Drosophila*, *Tribolium*, and *Oncopeltus*, and their expression in other species has suggested that this function is broadly conserved among arthropods (reviewed by Hughes and Kaufman, 2002b). However, what downstream events are directed by the gnathal Hox genes to produce mouthpart structure, and how might these downstream processes be modified in the evolution of divergent insect mouthpart morphologies?

The primitive mandibulate insect mouthparts consist of a gnathobasic mandible, paired maxillae with articulated palps and medial endites, and a medially fused labium with lateral jointed palps and medial endites (Figs. 1D–F). The mouthparts of *Tribolium* are of the primitive mandibulate type. The maxillary and labial palps and endites express *Dll* (Beermann et al., 2001). *dac* is expressed in intermediate domains, near the base of the endites as well as the proximal region of the maxillary palps (Prpic et al., 2001). Expression of *hth* appears in the base of the maxillary and labial appendages (Prpic et al., 2003). Antibody staining and in situ hybridization to orthologous sequences in *Acheta* (Abzhanov and Kaufman, 2000) as well as the mandibulate apterygote *Thermobia domestica* (Rogers et al., 2002) have revealed similar expression patterns for these genes. Therefore, in the primitive gnathal appendages, *Dll* is expressed along the telopodite (palp), while proximal leg markers are restricted to proximal regions: a pattern that greatly resembles the conserved pattern of expression in the legs of insects. Mutations in the *Tribolium* orthologs of the Hox genes *proboscipedia* (*pb*) and *Sex combs reduced* (*Scr*) produce transformations of the maxillary and/or labial palps to leg or antenna, respectively (DeCamillis et al., 2001). Thus, it is unlikely that the *Tribolium* Hox genes impose gnathal identity by regulating the PD domain genes, since their expression is not drastically different in the maxillae, labium, or legs.

However, it is likely that the evolution of the highly modified *Drosophila* proboscis ensued as *pb* and *Scr*

acquired negative regulatory influence over *Dll* and *dac*, thereby modifying the ancestral appendage developmental program. The *Drosophila* labial imaginal disc is specified by *pb* and *Scr* (Percival-Smith et al., 1997); *Scr* and *pb* act to repress PD domain genes, such as *Dll* and *dac*, as well as antennal genes such as *spalt* (Abzhanov et al., 2001; Fig. 4D). *Dll* repression is relieved by the absence of *Scr*, transforming the proboscis to maxillary palp identity (Fig. 4C). While in *pb* null mutations, *Dll* and *dac* are up-regulated causing a transformation to leg identity. However, this effect is indirect, as *pb* also acts to repress Hedgehog signaling, which leads to lower levels of *wg* and *dpp* activity (Joulia et al., 2005). This results in lowered expression of distal PD domain genes, such as *Dll* and *dac* in the wild type labial disc. Here, again, one sees the recurring theme of the interconnection between the Hox genes and the PD domain genes, as was evident in antenna/leg specification.

We have previously described PD domain gene expression and function from the hemipteran lineage (Angelini and Kaufman, 2004), whose members have mouthparts modified independently of the other insect orders. Hemiptera such as *Oncopeltus* have mandibular and maxillary appendages modified into thin feeding stylets, which are supported by a long-jointed labium that lacks palps (diagrammed in Figs. 4G and 5A). The mandibular and maxillary limb buds of *Oncopeltus* express *dac* and *hth* throughout their length (Fig. 4G). RNA interference has shown that *dac* is required for the maturation of the stylets, and its depletion does not appear to cause deletion of any PD domain. *Dll* expression is absent from the mandibular stylets, but appears throughout the maxillary limb buds. However, *Dll* RNAi causes no defect in either the mandibular or maxillary stylets. In the labium of *Oncopeltus*, *dac* expression appears in a small proximal domain later than in other appendage types while *Dll* is restricted to a distal region (Fig. 4H). RNA interference of *Dfd* transforms the stylets towards a partial antennal identity (Hughes and Kaufman, 2000; summarized in Fig. 5A), and in *Dfd*-depleted embryos, *Dll* becomes expressed in the distal half of the mandibular and maxillary appendages in an antenna-like pattern (Fig. 5C). The labium is transformed to leg or a mixed leg/antennal identity in *Oncopeltus pb* or *Scr* RNAi, respectively (Hughes and Kaufman, 2000). In both depletion backgrounds, expression of *dac* in the labial appendages appears in a broad intermediate domain indicative of leg identity (Figs. 5F–G). In the absence of clonal analysis, it is unclear whether these regulatory effects are direct, but these data indicate that the *Oncopeltus* Hox genes act to specify gnathal identities at or upstream of the PD domain genes. Again, this demonstrates the association of the Hox and PD pathways in specification of appendage identity.

In sum, the results of comparative analyses of mouthpart development from *Drosophila*, *Tribolium*, and *Oncopeltus* suggest that the evolution of novel mouthpart morphologies within the insects has involved Hox regulation of the PD

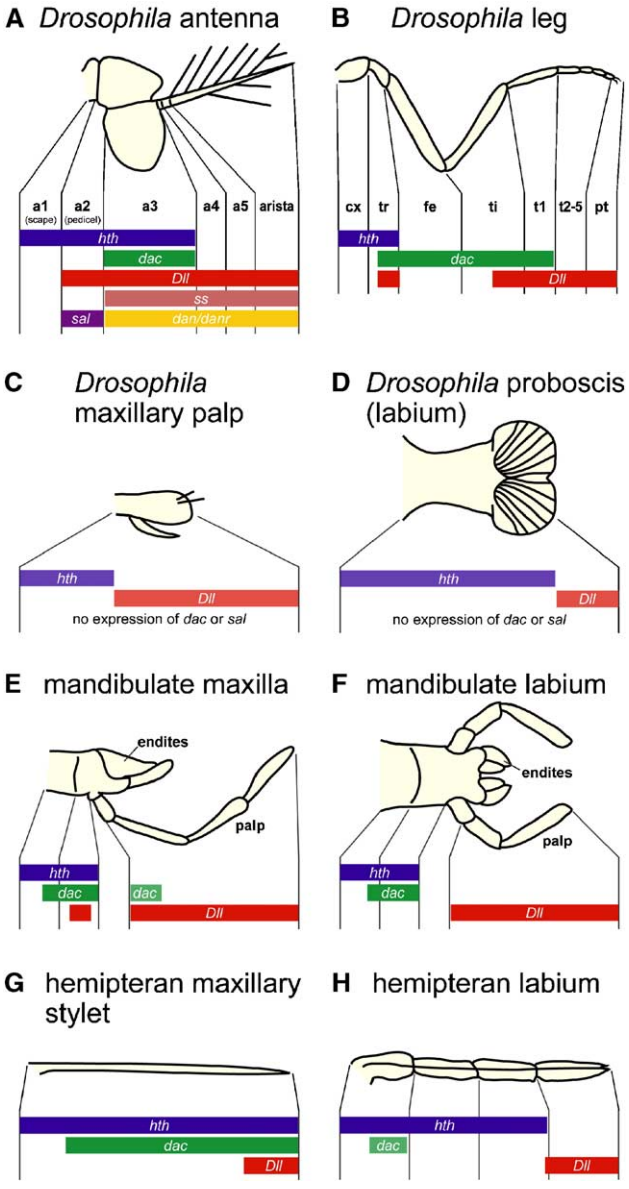


Fig. 4. Expression of PD domain genes varies in different appendage types. The (A) antenna and (B) leg of *Drosophila* are the best-studied appendage types. Expression in the *Drosophila* leg also seems to be representative of other insects. However, (C–D) the mouthparts of *Drosophila* are highly derived in morphology, and the activity of PD domain genes is mostly repressed in these appendages (see text). In contrast, gene expression in the ancestral mandibulate mouthparts (E–F) more closely resembles that of the legs (compare to B). Proximal expression of *Dll* in the maxilla correlates with the position of the medial endites. (G–H) Hemipteran mouthparts are highly modified in an independent lineage. (G) In the stylets, *dac* and *hth* are strongly expressed, while (H) in the labium, *dac* expression appears late and in a small domain. Thus, the expression of PD domain genes varies evolutionarily, as well as serially. Abbreviations: a1–a5, *Drosophila* antennal segment 1–5; cx, coxa; tr, trochanter; fe, femur; ti, tibia; t1–5, tarsi 1–5; pt, pretarsus. Transparency indicates weaker gene expression.

domain genes. This regulation may be direct, as in the case of *Dll* repression by *Scr* in *Drosophila*, or indirect, as manifest by the repression of *hedgehog* by *pb* in the fruitfly. However, data from *Tribolium* suggest that specification of primitive mandibulate mouthpart identity may not involve

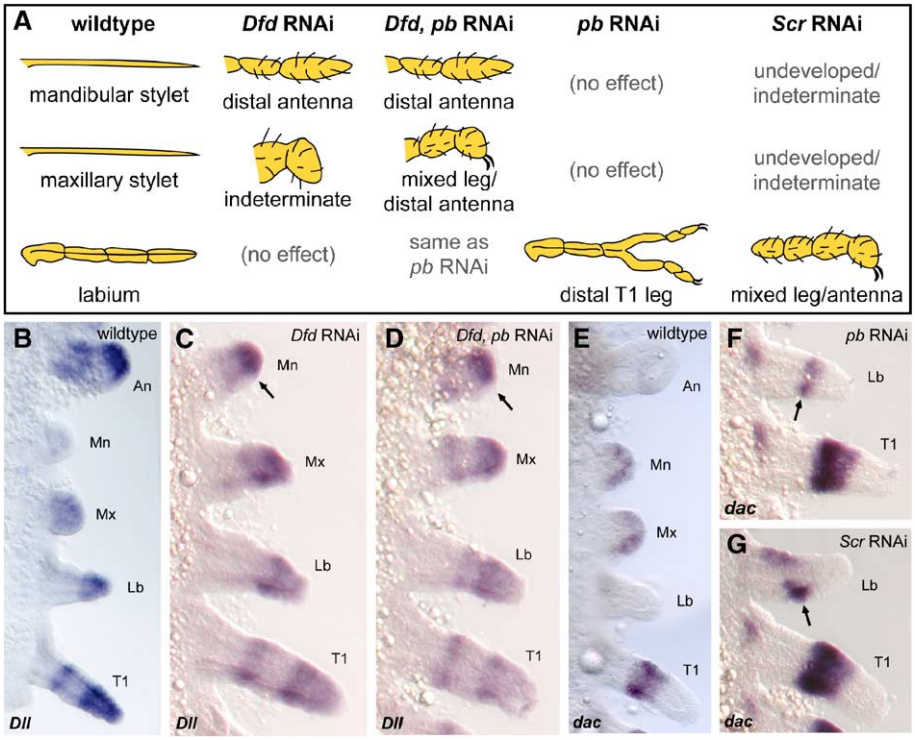


Fig. 5. Hox regulation of the PD domain genes *Distal-less* and *dachshund* in the milkweed bug, *Oncopeltus*. (A) A summary of Hox RNAi phenotypes in the mouthparts of *Oncopeltus*, modified from Hughes and Kaufman (2000). (B) Expression of *Dll* in the gnathal region of a wild type 72h-embryo. Note that the mandibular appendage lacks expression, while *Dll* is strongly expressed throughout the maxillary appendage. The T1 leg shows the ring-and-sock pattern characteristic of *Dll* expression in insect legs. (C) A *Dfd*-depleted embryo, showing that ectopic *Dll* expression appears in the mandibular appendage (arrow). (D) In *Dfd, pb* double RNAi, all gnathal appendages show a transformation toward an antenna-like pattern of *Dll* expression. (E) Wild type *dac* expression. In the labial appendages of (F) *pb*- and (G) *Scr*-depleted individuals, *dac* expression adopts a leg-like pattern. See the text for further discussion. Abbreviations: An, antenna; Mn, mandibular appendage; Mx, maxillary appendage; Lb, labial appendage; T1, prothoracic leg.

the regulation of PD domain genes. However, because of the phylogenetic position of *Tribolium* within the Holometabola, it is unclear whether this is a primitive trait retained from the ancestral insect condition, or whether it is secondarily derived from a state of Hox regulation ancestral to Eumetabola or all insects. While this later scenario may seem unlikely because mandibulate morphology is certainly the ancestral anatomical state for insects, *Antp* is required in the legs for leg-like expression of PD domain genes, and it is conceivable that gnathal Hox genes might also direct the expression of PD domain genes in similar patterns in the gnathos of primitive insects, such as the Orthoptera. Resolution of this issue will require clonal analyses in the beetle, as well as the investigation of additional insect groups with primitive gnathal appendages, such as the Orthoptera, and could benefit from the analysis of additional derived morphologies, such as those of the Lepidoptera.

Appendage allometry

Among insects, allometric variation in the size of appendages is extensive. The overall size of an individual insect's body is thought to be controlled by hormonal cues; however, the size of appendages relative to the body and to other limb types appears to be a complex matter (reviewed by Stern and Emlen, 1999). From observations and experiments in the *Drosophila* wing, it is thought that the size of an appendage does not depend simply on the number or size of cells. Rather, ontogenetic allometry appears to be regulated across developmental fields, such as the wing disc.

Allometry is also a highly malleable trait in evolution. Related species often show static allometric variation in particular organs, especially appendages (e.g., Huxley, 1932; Thompson, 1917). As an example, we have examined the length of the labium relative to body length among milkweed bug species of the genus *Oncopeltus* (Fig. 6). Individuals of this genus feed on various herbaceous plants throughout the neotropics. In another hemipteran, the soapberry bug *Jadera haematoloma*, divergent adaptation to separate host plants appears to have lead to drastic variation in mouthpart length among geographically separated populations (Carroll et al., 1997). Thus, the relative length of gnathal appendages appears to be easily modified in this group of insects.

We have found evidence that during larval development in *O. fasciatus*, levels of *Dll* expression can influence the length of the gnathal appendages. Embryonic or maternal injection of *Dll* dsRNA in *Oncopeltus* causes truncation of

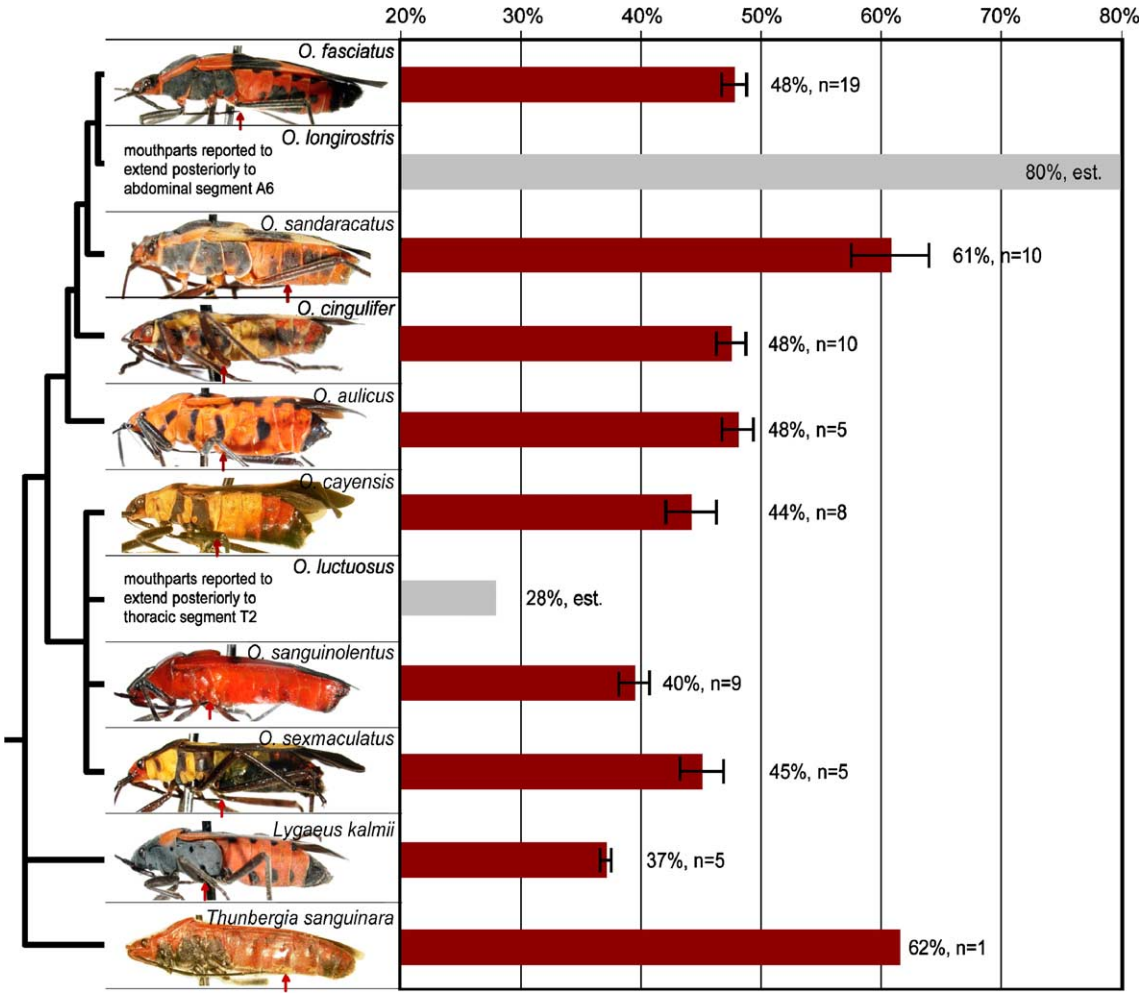


Fig. 6. Mouthpart allometry in *Oncopeltus* species. Relative mouthpart length was measured as the linear distance from the anterior-most point of the head capsule to the distal tip the labium, divided by the distance from the anterior of the head capsule to the posterior of the hind wings. This measurement of body length avoids variation due to differences in the preservation and retraction of the abdomen. Brackets denote standard error of the mean. Mouthpart length within species did not vary significantly among males and female. Estimates of labium length from *O. longirostris* and *O. luctuosus* are based on descriptions by O'Rourke (1979). The phylogenetic relationships indicated for these species are based on chromosome structure (O'Rourke, 1979) and external morphology (Slater, 1992).

the distal podomeres (Angelini and Kaufman, 2004). However, no appendage truncations were produced in juvenile stage RNAi. Instead, the labium was reduced in the next instar (compare Figs. 7A and B). Reduction in length also appears to be uniform along the PD axis, not concentrated to distal podomeres. (As a control, we preformed juvenile stage RNA interference of another PD domain gene, *dac*, which produces defects in stylets of the next instar,[2] but no change in appendage length relative to nonspecific control dsRNA injections.) These data suggest that during larval development, PD domain genes are no longer required to maintain the identity of limb podomeres; however, *Dll* does seem to function in the regulation of appendage length at each molt. It is tempting to speculate that variation in labium length between *Oncopeltus* species may result from differences in *Dll* activity, possibly as a result of *Sp8* regulation (see below). However, because Dll RNAi does not affect the stylets, this scenario would require that a separate mechanism regulate the length of these appendages. This example serves to illustrate the complex genetic means by which allometry may be controlled.

The hindlegs of insects are frequently larger than the more anterior legs. This increased size correlates with the expression of the Hox gene *Ultrabithorax* (*Ubx*) in the developing T3 legs of *Drosophila* (Akam, 1983; White and Wilcox, 1984). Among other insect species, the extent of *Ubx* expression, as assayed with an antibody against the

[2] In Hemiptera, the mandibular and maxillary stylets are shed at each molt and re-patterned in coiled invaginations called the retortiform organs (Parsons, 1964). *dac* nymphal RNAi results in a failure of the stylets to evert from the retortiform organs (54%, 7 of 13 surviving injection). This is similar to defects in the embryonic mandibular and maxillary appendages produced through embryonic and maternal *dac* RNAi. Therefore, this function in the development of the stylets appears to be retained for *dac* at each developmental stage.

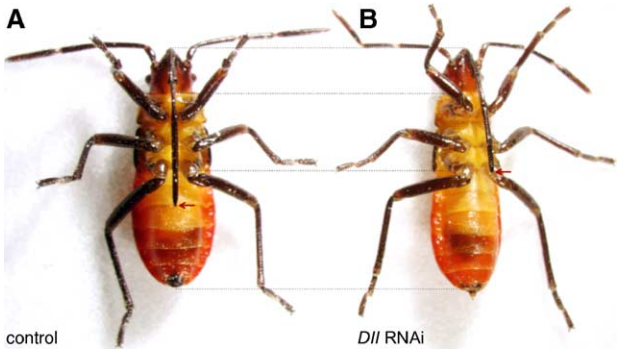


Fig. 7. RNA interference of *Dll* during juvenile instars in *O. fasciatus* results in a reduction in labium length in the next instar. Shown here are two third instar nymphs photographed side-by-side. Both were injected in the second instar with dsRNA encoding (A) GFP as a nonspecific injection control or (B) a *Dll* cDNA fragment (Angelini and Kaufman, 2004). Dotted lines indicate that the sizes of the body and specific body regions were not affected. Nymphs were injected in the second or third instar as described for *Tribolium* larvae by Tomoyasu and Denell (2004), and scored after molting. Of those injected individuals who survived molting, 55% or 6 of 11 showed a drastically reduced labium length, relative to control injections, which showed no affect. The labium is also foreshortened in relation to the stylets, which do not have a requirement for wild type *Dll* activity during embryonic development (Angelini and Kaufman, 2004). Changes were not observed in the length of antennae or legs, and it is unclear why the labium is particularly sensitive to *Dll* depletion.

Ubx and Abdominal-A proteins, prefigures and correlates with the relative size of the hindlegs (Mahfooz et al., 2004). The function of *Ubx* in leg allometry has been examined by Stern (2003) through clonal analysis in *Drosophila*. Clones lacking *Ubx* activity were autonomously reduced in cell size but produced nonautonomous reductions in the length (but not width) of the leg. However, reduction in leg length due to reduction in clonal cell size was partially compensated for by nonautonomous affects. Interestingly, nonautonomous changes were confined within podomeres. Conversely, ectopic clones of *Ubx* in the pupal T1 and T2 leg discs resulted in an increase in leg size comparable with wild type T3 legs. These results confirm that cell size and number are somehow regulated across podomeres. While *Ubx* appears to influence leg size, it evidently does so through several mechanisms. Stern (2003) concludes that allometry may be controlled by a number of complex mechanisms, which would allow greater evolutionary flexibility in the selection of appendage size.

Another mechanism of ontogenetic allometry in insects may be the regulation of *Sp*-class zinc-finger transcription factors (so named because they were first isolated from vertebrates using chromatography columns with Sephacryl and phosphocellulose; Philipsen and Suske, 1999). *Sp1* and *buttonhead* (*btd*) are required for specification of the *Drosophila* leg primordia. Later in leg development, these genes are required for the antennae and legs to attain their normal length. Evidence for this conclusion derives from the fact that RNA interference of *Sp1* and *btd* in *Drosophila* imaginal discs does not delete podomeres, but reduces their size, albeit preferentially in distal segments (Estella et al., 2003). The *Drosophila Sp1* orthologue has been examined in *Tribolium*, where it is called *Sp8*, due to sequence homology with its apparent vertebrate orthologue *Sp8* (Beermann et al., 2004). RNA interference of *Tribolium Sp8* produces a similar foreshortening of the appendages. While distal podomeres are more seriously reduced, they are not deleted as in *Dll* mutants. Interestingly, there is a correlation between the expression patterns of these *Sp* genes and the size of serially homologous appendages. In *Tribolium* and *Drosophila*, the *Sp* genes are expressed in ring domains at various PD levels. *Tribolium Sp8* is expressed throughout the shortest appendages, the labrum and labium, while in the antenna and maxilla, two ring domains appear. The legs, which are the longest appendage type, bear four rings of *Sp8* expression (Beermann et al., 2004). In *Drosophila*, *Sp1* and *btd* expression overlap in an intermediate ring in the antennal disc, and in two rings in the leg discs. Thus, the number of *Sp* domains in these species correlates with the ontogenetic and evolutionary size difference of appendages. However, it is unclear how the number of *Sp* expression rings might influence appendage growth. One possibility is via *Dll*: *Dll* is a known *Sp* target early in *Drosophila* ventral primordia, and distal structures are more severely reduced when *Sp* genes are depleted in both species.

Conclusions

In conclusion, it appears that the specification of the limb primordia and axes appears to have been less well conserved than later processes. While taxonomic sampling is very limited, the roles of *wg* in specification of limb primordia and axes appears to have evolved within the Holometabola, and *dpp* appears to have been recruited to a limb function since the divergence of *Tribolium* and *Drosophila*. Therefore, insects appear to possess a great diversity in the early patterning events of the limb primordia; however, functional data from *Gryllus* and *Oncopeltus* suggest that, ancestrally, *wg* and *dpp* were not involved in specification of limb primordia or axes.

Once the limb primordia have been established, the expression patterns of most PD domain genes appear to be well conserved in the legs of insects. *Dll*, *dac*, and *hth* are expressed in distal, intermediate, and proximal domains. *Dll* and *dac* are exclusive early but come to overlap at later stages. To the extent that these genes have been functionally tested, there appears to be a conserved requirement for them in the patterning of their respective domains.

What might be the reason for conservation of the PD leg domains? The function of the insect leg in walking is tied to the anatomical specialization of its podomeres (Daly et al., 1998; Delcomyn, 1985). As such, selection may act to maintain some developmental mechanisms in the leg, such as the distinct domains established through the mutually repressive interactions of *Dll*, *dac*, and *hth*. This possibility

could be tested indirectly through examination of the expression and interactions of these genes in insect lineages where the biomechanical function of the thoracic appendages is not in walking. For example, in the aquatic hemipteran family Notonectidae (the backswimmers), the tibia and undivided tarsus are elongated and flattened into an oar. Aquatic beetles of the family Dytiscidae have hindlegs independently modified for swimming: the tibia and tarsi are roughly equal in size and covered in bristles to form a "swimming brush" (Daly et al., 1998). The divergent biomechanical function of the "hindlegs" in these groups (Gittelman, 1974) and their more homonymous morphology along the PD axis, predicts that the ancestral states of PD domain gene expression and interaction may have been modified in these groups.

Variation in PD domain gene expression patterns and timing (and presumably interactions) seems to correlate with derived mouthpart morphology. As we have described, mandibulate mouthparts are similar to legs in their expression of genes such as *Dll*, *dac*, and *hth*. In species with divergent mouthpart morphologies, such as *Drosophila* and *Oncopeltus*, the expression and developmental function of these genes can be quite different in the gnathal appendages.

Finally, our understanding of the genetic basis of allometric variation in appendages is still limited. A number of genes, such as Hox and *Sp*-class transcription factors, have been implicated in size specification at various developmental levels. However, it remains unclear how the activity of these genes specifies appendage size, or what upstream regulatory inputs govern their allometric roles.

Future directions

Numerous questions remain to be investigated in the study of insect appendage development. Throughout this review, we have attempted to highlight ambiguous and tentative issues. However, several broad issues are also of concern.

To achieve a complete understanding of how genes specify the identity of domains along the limb PD axis, it will be necessary to determine the downstream targets of regulatory networks governed by genes such as *Dll* and *dac*. Currently, very little is known about whether different appendage types employ qualitatively different effector genes, or whether the same genes are simply activated at slightly different levels, times, or locations in different appendages. Neither is it known whether the number of targets shared by different appendage types might be large or small, or whether there is any degree of feedback upon the known regulators. *Drosophila* genetics and genomic studies will provide useful tools to this end.

Drosophila remains the preeminent model of developmental and genetic analyses, and a more detailed understanding of morphogenesis in this species is still necessary. In particular, this genetic system may provide new insights into the specification of size, perhaps through the examination of hormonal activity in specific genetic backgrounds.

To understand how these developmental mechanisms may be modified during evolution, it will be necessary to extend the examination of gene expression and function into more non-model species. In particular, those species with divergent morphology may help to distinguish commonalities and eccentricities of appendage patterning among insects. By pursuing forward genetics in species such as *Tribolium*, it will be possible to identify genes not present in *Drosophila*. By subsequent comparisons in other species, it should eventually be possible to define the ancestral mechanisms of appendage patterning and describe the molecular events that have accompanied the evolution of limb morphology.

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